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13. ABSTRACT *(Maximum 200 words)* Estrogen has been shown to be involved in the progression of breast cancer and the estrogen receptor (ER) has been implicated in reproductive cancers. Our laboratory would like to understand how the structure of the hormone binding domain (HBD) of the ER changes when it is "activated" by estrogens and anti-estrogens. Since the binding of estradiol to its receptor ultimately leads to diverse biological responses to the hormone, we would like to investigate the response of the receptor to a series of ligands and illucidate the molecular basis for their functional differences. This proposal will characterize the critical substrate induced conformational changes in the HBD by incorporating fluorine labels into two recombinate constructs and performing fluorine nuclear magnetic resonance (NMR) studies. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor and will compare these changes to those induced by "environmental" estrogens. The purpose of this proposal is to provide and understand the conformational changes in HBD which will shed light on the molecular events of substrate binding, transcriptional activation and the role of environmental estrogens in receptor function.

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Linda Luck

PI - Signature Date

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INTRODUCTION

The biological importance of estrogen is noted by the number of disease states associated with altered production of estrogen or estrogen like materials. Estrogen has been shown to be involved in the progression of breast cancer and the estrogen receptor (ER) has been implicated in reproductive cancers. One early step in the process of transcriptional activation by estradiol is the production of conformational changes in the ER upon ligand binding. Our laboratory would like to understand how the structure of the hormone binding domain (HBD) of the ER changes when it is "activated" by estrogens and antiestrogens. Since the binding of estradiol to its receptor ultimately leads to diverse biological responses to the hormone, we would like to investigate the response of the receptor to a series of ligands and elucidate the molecular basis for their functional differences. This proposal will characterize the critical substrate induced conformational changes in the HBD by incorporating fluorine labels into two recombinant constructs and performing fluorine nuclear magnetic resonance (NMR) studies. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor and will compare these changes to those induced by "environmental" estrogens. **The purpose of this proposal is to provide an understanding of the conformational changes in HBD which will shed light on the molecular events of substrate binding, transcriptional activation and the role of environmental estrogens in receptor function.**

Conformational changes in the estrogen receptor. ER is a member of the steroid receptor super family which includes proteins whose functions are to bind small hydrophobic ligands and mediate transcriptional activity within the nucleus.[1,2] These receptor proteins have four distinct actions which modulate biological activity: (1) binding hormone, (2) forming multimeric complexes, (3) binding to sequence specific DNA, known as hormone response elements and (4) modulating transcription. As a consequence of this action DNA synthesis is changed as well as RNA and proteins necessary for cell proliferation. Studies have revealed distinct functional domains in these proteins which include (1) the N-terminal domain which is postulated to be involved in transcriptional enhancement (2) a DNA binding domain, and (3) HBD at the C- terminal end which is responsible for high-affinity binding of steroids. Participation of accessory proteins have been the subject much investigation and have indicated that ER function depends on association with other proteins.[3] In the absence of estrogen, ER is bound in a large molecular complex which includes heat-shock protein 90 (HSP90), HSP70, P59, and other proteins.[4,5] Binding of estrogen to ER releases the monomeric receptor from the complex by inducing a conformational change in the HBD. This conformational change is key to the cascade of events leading to transcription and subsequent protein production.[6] In addition, conformational change is important in dimer formation of ER which allows cooperative ligand binding lowering the ligand concentration range for full action of the protein.

The HBD is of great interest because it contains the regulatory actions of the protein. It is the interaction of the hormone-receptor complex with response elements that can result in induction

or repression of transcription. Chimeric constructs have been made with unrelated proteins such as the *myc* oncogene product which show hormone regulation of the fused gene product. This suggests that the HBD alone is responsible for its own action of ligand binding and conformational change. The interplay between the ligand and the HBD may carefully select how the biologies are manifested.

Many studies have proposed a conformational transition upon binding of estrogen. At the commencement of this work there was a dearth of information concerning the molecular basis of these changes in the receptor itself. Since our studies began, the X-ray structure of the HBD has been solved with 17Beta-estradiol (E2) and raloxifene (RAL) in the binding pocket.[7,8] This work has shown conformational differences in HBD with these two diverse ligands and demonstrates the need for further work on this protein in the solution state. According to these studies the HBD is comprised of a 3 layered antiparallel alpha helical sandwich. The wedged shaped molecule accommodates a sizeable ligand binding cavity. The HBD in the crystal form is a non crystallographic dimer with a head-to-head arrangement leaving the end termini of each monomer at opposite sides of the whole molecule. 15% of the surface is involved in the dimer interaction mainly through contacts on a helix designated as H11.[7] Structural differences between RAL and E2 bound HBD include an extensive change in an alpha helix (H12) which has been established to be essential for transactivation.[9] In the E2 complex the H12 fits on top of the binding cavity forming a lid, sealing the ligand in the pocket. In contrast the RAL does not permit the H12 to cover the cavity due to its long side chain and does not form a competent transactivational site since the overall topography of the surface has changed. This work supports our initial premise that the conformational changes in the receptor are key to the biological events in the cell.

Our underlying premise in this proposal is that conformational changes in the ER are crucial to the function of the receptor and its interaction with associated proteins and nucleic acids. The initial conformational change upon ligand binding may play the most important role regulating the course of action of the receptor in subsequent transcriptional events. We hypothesize that the initial structural form of the liganded receptor is the pivot point for the future events within the nucleus. We will examine whether estrogens and antiestrogens produce similar conformational effects on the receptor. We will compare these changes to those induced by "environmental" estrogens.

BODY

SYSTEM SET UP The first goal in this study will be to produce recombinant HBD with fluorine labels for study by NMR. As reported last year we worked with two constructs. The first construct which contains the fusion protein glutathione-S-transferase in tandem with the amino acids 282-595 of the hormone binding domain of the human estrogen receptor (GST-HBD) was made in the Pgex2T vector from Pharmacia. The second construct (GG) was obtained from Geoff Greene's laboratory [10,11] which contains the residues 282-595 of the HBD in the vector Pet23d from Novagen. Both constructs were transformed into nonauxotroph *E. Coli* cell lines and produce milligram quantities of unlabeled HBD in rich media.

GST-HBD PROTEIN. Our first goal is fluorine label the protein. To accomplish this the GST-HBD construct was transformed into the cell lines W3110, KA197 and AT2470 which are Trp, Phe and Tyr auxotroph cell lines, respectively. The GST-HBD plasmid transformed in the W3110 cell line produced protein with 5F-Trp labeling. There are four Trp residues in both the glutathione portion of the protein and the HBD (292, 360, 383, 393). Using a 5:1 ratio of labeled trp to unlabeled trp we were able to get approximately 5 g of pellet per liter of bacteria. Protein purification was accomplished by means of a glutathione sepharose column and elution with 20 mM glutathione. This proteolysis of the HBD under mild conditions results in a stable protein core of mass 25-30 ka which binds ligand and has been observed in Greene's laboratory[11]. Further studies on this construct showed that the protein after storage did not hold up. The fluorine labels were detrimental to the protein integrity and we were not able to successfully combine preparations for NMR studies. Our initial spectra were obtained by using fresh preparations of protein and these data did not give good separation of peaks for assigning peaks and further evaluation of the conformational changes with other fluorine labels. In addition, the protein was broken down by the use of thrombin due to a secondary thrombin site which also cleaved when we attempted to cleave the GST from the HBD. Because of this we are unable to get HBD released from the tandem protein. Thus, we have abandoned this particular construct to produce HBD and this approach to the project. On a positive note, we have been successful in terms of the NMR since no one has been able to see signals from such a large protein complex a manuscript addressing this is in progress. **We have shown by this work that conformational changes do occur with addition of ligand to the HBD protein.**

HBD PROTEIN. The protein produced by the GG construct is isolated by the use of a estradiol sepharose column provide by the Greene laboratory. The HBD is eluted with estradiol or other substances that will compete with estradiol for the binding site of HBD. The protein shows a doublet around 33kDa on SDS-Page gels and a positive western blot using AE320 or 311 antibodies from Neomarkers Inc. The HBD is soluble up to 20 mg/ml or .2-.6 mM which is sufficient for all of our NMR studies. The original HBD obtained from Greene contained the residues from 282-595. This protein broke down to a series of smaller peptides during the process of purification. This was probably due to protein instability caused by the urea and the fluorine labels. We have since obtained a new construct which spans 297-554 (sGG). Our attempts to transform sGG into auxotroph cell lines, W3110, AT2471 and KA197 for Trp, Tyr and Phe respectively did not produce viable colonies. We have found that other constructs using pET vectors were also incompatible for transformation with these cells lines. We have been forced to search for alternative methods for obtaining fluorinated proteins from non-auxotroph cell lines. Two such methods are (1) addition of large concentrations of fluorinated amino acids to cultures to flood the cells or (2) adding glyphosate which inhibits aromatic amino acid synthesis and adding the appropriate fluorinated amino acid in addition to the unlabeled amino acids necessary for growth.[12] Monsanto has kindly provided us with gram amounts of glyphosate for our experiments so our costs are not prohibitive. We have performed growth curves with minimal media, glyphosate and the various labeled and unlabeled aromatic amino acids to quantitate growth and protein production. Our studies so far have shown that glyphosate with added L-Tyr, L-Phe and 5F-Trp does not inhibit growth of the bacteria and we have been able to get

protein production from the plasmid under these conditions. As yet, many of our preparations did not show fluorine signals in the NMR experiments. Our protein samples were not as soluble as those predicted in the Greene lab. We have also changed our purification protocol to use ammonium chloride to prevent carbamylation of the lysine residues.[8]

Because of the lack of NMR signals in some of our preparations, we were forced to deviate from our original focus to pursue studies to find a way to evaluate the amount of fluorine in the proteins. In past experimentation on bacterial proteins, we used an internal standard in the NMR experiment itself to provide a measure of the amount of fluorinated protein. This approach was not feasible with the ER proteins. We needed a way to monitor the amount of fluorine in small quantities of expressed protein so that we could alter methods of preparation on a small scale before making large cultures for NMR. We spent several months perfecting such a method to provide a measure of the amount of fluorine incorporation using bacterial proteins and soluble tissue factor in collaboration with the Ross laboratory and the W.Alton Jones Cell Center Mass Spectroscopy Laboratory. One paper has been submitted to Protein Science which is included with this report and one paper is in progress to be submitted to Analytical Biochemistry. These methods are now being used to monitor our fluorine incorporation for the preparations of HBD.

MAL-HBD PROTEIN. The removal of estradiol from the binding pocket of the GG and sGG proteins has been less than successful. In order to show conformational change in the protein before and after ligand binding we must have an empty binding pocket in HBD. We have decided to pursue yet a third construct to obtained protein that is stable and can be prepared without ligand in the binding pocket. We also felt there was some controversy as to the amount of urea used in the preparation of GG and sGG.By using two different protein preparations we can evaluate the use of urea as an agent for purification. Mark Brandts has provided us with the construct of the HBD (300-551) which is placed in tandem with the maltose binding protein. These constructs, pER304 and pER336 express wild type HBD and have been transformed into our auxotroph cell lines CY15077ea2 (trp auxotroph) KA197 (phe auxotroph), AT2470 (tyr auxotroph) and BL834 (met auxotroph). Production of the protein in these cell lines is observed on a gel and the full purification has been completed for the trp auxotroph line. An added bonus is that all of the trp to tyr mutants have been made and are available from Mark's laboratory. [13] This construct is also smaller and can be used to look at the dimers along with the monomers in solution.

We have also made trifluoromethionine in our laboratory for labeling the methionine residues with the guidance of John Honek, University of Waterloo. [14] There are 14 met residues in the pER 304 and 336 which can be labeled by fluorine. This will increase the areas in the protein which we can observe by the fluorine NMR methods.

Philip Cole from Rockefeller University has also provides us with 2F-Tyr which he has synthesized in his laboratory.[15] This fluorinated analogue has the nearly the same pKa as normal Tyr and should prove to be a better labeling agent than the 3F-Tyr currently available through commercial sources. The pER constructs are in the tyr auxotroph lines and work is in progress to

purify these proteins.

We have been granted an extension of a year to provide more work on this project. During the month of January 1998 an ice storm hit our area. At this time the electricity went out in the laboratory and all of the refrigerators and freezers were without power for days. All of our protein preparations that were waiting for NMR runs were ruined in this thaw. We had to retransform many of the cell lines that were in frozen permanents. This was a very discouraging set back.

CONCLUSIONS

Using two plasmid constructs for the hormone binding domain of the human estrogen receptor we have found that we can produce protein that is functional in binding estradiol as that found *in vivo*. In addition we have been able to label this protein with 5F-Trp in order to obtain ^{19}F NMR spectra. The results from the NMR spectra from GST-HBD labeled with 5F-Trp shows conformational changes in the hormone binding domain of the estrogen receptor when estradiol binds to the pocket. In addition the protein shows a more rigid conformation when the estradiol is present. We have shown that fluorine NMR can be used to investigate the binding of fluorinated ligands similar to DES. We have concluded that the GST-HBD construct has very limited use due to its large size. The HBD protein GG and sGG can be fluorine labeled and purified by the estradiol column. Our initial spectrum shows shows four peaks for the four Trp residues for HBD, two are overlapped suggesting that the environments about two tryptophan may be the same in the structure. The urea in the purification scheme has cause some problems in the final preparations. We are adjusting the conditions for the fluorine labels. We have also included in the study a new construct which is the Maltose binding protein in tandem with the HBD which transforms into the auxotroph cell lines and can be purified without the estradiol in the binding pocket.

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^{19}F NMR of 5-Fluorotryptophan in Soluble Human Tissue Factor: Comparison with Results from Tryptophan Difference Fluorescence and Absorption Spectroscopies

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ABSTRACT

^{19}F nuclear magnetic resonance (NMR) spectroscopy was used to investigate the local environments of the four tryptophan (Trp) residues of the extracellular domain of human tissue factor (sTF) by using 5-fluorotryptophan (5F-Trp) as a probe. sTF has two sub-domains, Domain I which contains three Trp residues and Domain II which contains one Trp residue. The ^{19}F NMR assignments to specific Trp residues were based on comparison of the wild type protein spectrum with spectra of single Trp-to-Phe replacement mutants. Replacement of the Trp residues by 5F-Trp had no effect on the biological activity of either the wild type or mutant proteins. Solvent-induced isotope-shift (SIIS) and paramagnetic broadening experiments were used to assess solvent accessibility at each Trp site. The information obtained from ^{19}F NMR was compared with that obtained previously from absorption and fluorescence difference spectra of Trp using the same mutations. ^{19}F NMR showed that the single Trp-to-Phe mutations in Domain I caused subtle changes in the local environments of the two Trp residues remaining in that domain. These changes, however, had no effect on function. Finally, the paramagnetic broadening and SIIS experiments provide information about indole ring solvent accessibility that is complementary to that obtained from absorption and fluorescence spectroscopies.

INTRODUCTION

Following tissue damage, the extrinsic pathway of the blood coagulation cascade is initiated by the interaction between Tissue Factor (TF), a membrane-bound glycoprotein, and Factor VII/VIIa (VII/VIIa), a serine protease that circulates in the blood (see reviews by Bach, 1988; Nemerson, 1988, 1995; Edgington et al., 1991; Davie, 1995; Østerud, 1997). The active enzyme, VIIa, is generated from zymogen VII by a specific internal cleavage, forming a 152-residue light chain and a 254-residue heavy chain. TF, a member of the cytokine receptor super-family, is a 263-residue polypeptide that consists of an extracellular domain (residues 1-219), a single transmembrane domain (residues 243-263), and a cytoplasmic domain (residues 243-263). In the absence of TF, VIIa has virtually no proteolytic activity towards its zymogen substrates, Factor IX (IX) and Factor X (X). Thus, with respect to the coagulant activity of VIIa, binding to TF is obligatory, and TF serves as an essential cofactor for VIIa (Nemerson & Gentry, 1986).

The association between TF and VIIa occurs at a membrane surface, and involves protein-protein and protein-lipid interactions. To investigate the features of the protein-protein interaction, we have used the soluble, truncated extracellular domain of TF (sTF), expressed in *Escherichia coli* (*E. coli*). Although sTF lacks the transmembrane and cytoplasmic domains, it retains procoagulant activity when phospholipids are present (Waxman et al., 1992; Neuenschwander & Morrissey, 1992; Fiore et al., 1994). We have shown that in the absence of lipids, sTF and VIIa form a binary complex with an equilibrium dissociation constant, at 20°C, of about 1 nM (Waxman et al., 1992). Hydrodynamic studies by analytical ultracentrifugation and time-resolved fluorescence anisotropy

(Waxman et al., 1993a), as well as by x-ray and neutron scattering (Ashton et al., 1995), have shown that both proteins and their complex are highly asymmetric. They show also that a major conformational change occurs in VIIa upon binding sTF, specifically a loss in segmental flexibility. The recent x-ray crystal structure of the complex between sTF and active site-inhibited VIIa, by Banner et al. (1996), reveals important details of the asymmetry and the intermolecular interaction (see Figure 1). In addition, comparison of the conformations of the main chain α carbons of sTF alone, obtained from the crystal structure by Muller et al. (1994), with those of sTF bound to VIIa, shows no significant differences (Δ r.m.s. = 0.5 Å) in the main chain conformation of sTF (Banner et al., 1996). This suggests that the extracellular domain of TF provides a template to stabilize the catalytically efficient conformation of VIIa, and that conformational changes in TF are restricted mainly to rearrangements of side chains.

To elucidate the subtle conformational changes that might occur when sTF interacts with VIIa, we are using ^{19}F NMR spectroscopy. The chemical shifts of ^{19}F are particularly sensitive to changes in the local electrostatic fields and changes in van der Waals contacts (Gerig, 1994; Danielson & Falke, 1996). As a result, the chemical shifts are useful for observing changes in the local environment. Moreover, the molecular weights of sTF and its complex with VIIa, which are about 25,000 and 74,000, respectively (Waxman et al., 1993a), should pose no problem as molecules with molecular weights up to 100,000 can be investigated by this approach (Gerig, 1994; Danielson & Falke, 1996). Using the appropriate auxotrophic cells, recombinant proteins expressed in E. coli can be labelled with fluorinated analogs of tryptophan or phenylalanine. It is

important that the analogs be incorporated at sites that are sensitive to interaction. The x-ray crystal structure of the sTF:VIIa:inhibitor complex (Banner et al., 1996) shows that two of the four Trp residues in sTF, Trp 45 and Trp 158, are involved in direct intermolecular interactions with VIIa. First, Trp 45 forms an amide hydrogen bond with the carbonyl oxygen of Phe 275 of the VIIa protease domain – this is the only contact between the main chains of the two proteins – and the indole ring of Trp 45 packs against the side chain of Arg 277 of VIIa. Second, Trp 158 is involved in the main hydrophobic interaction with VIIa, which includes participation of Phe 31 in the Gla domain of VIIa and both Val 207 and the nearby Cys 186 – Cys 209 disulfide bridge of sTF. Thus, the Trp residues of sTF, replaced by F-Trp analogs, should be suitable for studying the interaction with VIIa, provided the resonances of the four F-Trp residues can be resolved and assigned.

In this paper we report the ^{19}F NMR spectroscopy and functional properties of sTF labeled with 5F-Trp. The resonance assignments for each 5F-Trp residue are based on differences between the ^{19}F NMR spectra of wild type and single-Trp replacement mutants. Also, solvent-induced isotope-shift (SIIS) and paramagnetic line broadening experiments were carried out to assess the solvent accessibility of the 5F-indole side chains. The ^{19}F NMR data for the wild type and mutant proteins are compared with those from our previous investigation based on Trp absorbance and fluorescence spectroscopy (Hasselbacher et al., 1995a), showing that the NMR and optical spectroscopies provide complementary information. In particular, the 5F-Trp ^{19}F NMR spectra reveal subtle perturbations in the local environment of the NMR probe that result from single Trp-to-Phe mutations within the same protein domain. The sensitivity of ^{19}F chemical shifts to subtle

structural perturbations, demonstrated from the mutant spectra, suggests that ^{19}F NMR will be a valuable tool for investigating the macromolecular interactions in the binary sTF:VIIa complex and ternary complexes with substrates or inhibitors.

MATERIALS AND METHODS

Reagents

5-d,l-Fluorotryptophan was from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI) and Acros (Pittsburgh, PA). Recombinant human VIIa was a generous gift from Novo Nordisk (Denmark), and factor X was purified from human plasma by the methods of Miletich, et. al., (1981) and Broze & Majerus (1980). 1,2-Dioleoyl-*sn*-glycero-3-phosphotidylserine and 1,2-dioleoyl-*sn*-glycero-3-phophotidylcholine were from Avanti Polar Lipids (Alabaster, AL). The chromogenic substrate Spectrozyme was from American Diagnostica Inc. (Greenwich, CT). Guanidinium chloride (Gdm·Cl) was from Heico Chemicals (Delaware Water Gap, PA). Deuterium oxide (D_2O), gadolinium (Gd), and diethylenetriamine-pentaacetic acid:gadolinium (III) dihydrogen salt dihydrate (Gd:DPTA) complex, were from Aldrich (Milwaukee, WI).

Expression and purification of wild type and mutant proteins

The wild type and mutant plasmids (W14F, W45F, W158F) were the same as previously described (Hasselbacher et al., 1995a). Following standard protocols (Sambrook et al., 1989), the plasmids were transformed into the *E. coli* tryptophan auxotroph CY15077ΔEA2 (Ross et al., 1997). The resultant strains were grown overnight at 37°C in 6 × 1 L of Terrific Broth

supplemented additionally with 4 mL/L of glycerol, and the cells were harvested by centrifugation. Each cell pellet from 1 L of culture medium was washed in 100 mL of M9 salts, and then resuspended in 1 L of M9 salts, supplemented with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.04% glucose, 1% casamino acids, 0.1% thiamine and 60 μg/mL ampicillin. The cultures were shaken for 1 hour at 37°C, then transferred to 30°C, supplemented with 50 mg/L 5F-Trp, and shaken for 30 additional minutes. Isopropyl-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce protein expression, and the cells were harvested by centrifugation after 4-5 hours of shaking.

The cDNAs for sTF, the Trp-to-Phe mutants, and one Trp-to-Tyr mutant were constructed with a leader sequence that directs the protein to the periplasmic space of *E. coli*. Previously, the proteins were purified from concentrated media after overnight induction at 20°C (Hasselbacher et al., 1995a). With the shorter induction period used here, most of the expressed protein is retained in the cells. Therefore, the proteins were released from the periplasmic space by osmotic shock using the following protocol: The cell pellets were re-suspended in 1 L of 30 mM Tris, pH 8, 20% sucrose, 1 mM EDTA, and stirred for 5 minutes at room temperature. The cells were pelleted by centrifugation and the supernatant was discarded. The pellet was re-suspended in 1 L of ice-cold 5 mM $MgSO_4$ and stirred for 5 minutes at 4°C (adapted from Snavely et al., 1989). The lysed cells were pelleted and discarded, and $(NH_4)_2SO_4$ was added to the supernatant to 65% saturation. The remaining purification steps were essentially as described by Waxman et al. (1992).

The yields of proteins expressed in the *E. coli* Trp auxotroph CY15077ΔEA2 (Ross et al.,

1997; Hasselbacher et al., 1995b) varied in a mutant-dependent manner. For example, the yields from 6 L of bacterial culture were about 65 mg for wild type sTF but only about 1 mg for mutant W14F and much less for mutant W25Y. Unfortunately, the quantity of W25Y recovered was insufficient for NMR. It should be noted that the same levels of protein expression were observed, whether Trp (Hasselbacher et al., 1995a) or 5F-Trp (this study) was used for protein synthesis. The relatively low expression of W14F and W25Y suggests that protein folding is affected when these Trp residues are replaced with amino acids that have smaller side chains. According to the x-ray crystal structure (Muller et al., 1994; Harlos et al., 1994), Trp 14 and Trp 25 are buried within the protein matrix of Domain I, and Trp 25 is at the center of extensive packing interactions.

Assessment of analog incorporation

Incorporation of F-Trp analogs is generally less than 100%, and depends upon the analog and method of incorporation (Danielson & Falke, 1996). Two approaches were used to assess the level of 5F-Trp incorporation into wild type and mutant sTF proteins. The first involved fitting absorbance spectra of the proteins, denatured at neutral pH in 6 M Gdm·Cl, by LINCS analysis (Waxman et al., 1993b) using the absorption spectra of N-acetyl-Trp-amide, N-acetyl-Tyr-amide and 5F-Trp[1] as basis spectra. The criterion for a satisfactory LINCS analysis is a minimum χ^2 statistic and recovery of the correct Tyr-to-'Trp' ratio ('Trp' = Trp + 5F-Trp); the fit should

[1] To recover the correct Tyr-to-Trp ratio from LINCS analysis of proteins or peptides of known composition containing Trp analogs such as 5-hydroxytryptophan or 7-azatryptophan, it was necessary to block the α-amino groups of the analogs. However, free 5F-Trp at neutral pH in 6 M Gdm·Cl provided a satisfactory absorbance basis spectrum for 5F-Trp-containing sTF.

represent the absorption of Tyr, Trp and 5F-Trp residues in the denatured protein spectrum. Then the percentage of 5F-Trp incorporated is determined from the mole ratio of 5F-Trp to total 'Trp'. Absorption spectra were measured at room temperature using a Hitachi U-3210 spectrophotometer. The wavelength region used for analysis was between 270 and 340 nm to avoid interference from phenylalanine absorption, and the minor extinction contributions from the two disulfide bridges in sTF were ignored. According to LINCS analysis, 5F-Trp incorporation in the wild type protein is essentially 100% while it varied from 77 to 95% for the mutants (Table 1).

Mass spectrometry provides a direct assessment of micro-heterogeneity of 5F-Trp incorporation by revealing proteins where all, some, or none of the Trp residues are replaced by 5F-Trp. Electrospray mass spectrometry (ESMS) and liquid chromatography ESMS (LC ESMS) were performed at the W. Alton Jones Cell Science Center, using Perkin-Elmer Sciex API 300 triple quadrupole mass spectrometer (Concord, Thornhill, Ontario Canada) fitted with an articulated ion spray plenum and an atmospheric pressure ionization source. For infusions in positive ion mode, spectra were acquired at an orifice potential of 55 V over a scan range of 400 - 2600 m/z using 0.25 amu steps and a total scan time of 6.2 s. Between 25 - 35 spectra were taken by multi-channel acquisition for each sample infused. Mass calculations were performed with the BioSpec Reconstruct program in the Perkin-Elmer Sciex software package.

Estimates of total analog incorporation were obtained from a mass spectrum by comparing relative peak heights of each mass species, and taking into account the number of incorporated

fluorine atoms represented by that mass. The estimates obtained are similar to those determined by LINCS analysis (Table 1). For example, the mass spectrum of a three-Trp protein might display four mass peaks representing protein containing none (wild type), one, two, and three atoms of fluorine, respectively. To estimate the percent 5F-Trp incorporation, the peaks representing each species were identified by their appropriate masses, and higher mass peaks resulting from salt adducts were ignored. Assuming that the sum of the peak heights of appropriate mass is proportional to the total expressed protein, the *mole fraction* of each species is the peak height of each appropriate mass divided by the sum that represents the total mass. The percent incorporation of 5F-Trp, %I, in the total protein then is given by

$$\%I = 100 \times \sum_{i=0}^{i=n} \alpha_i f_i \qquad (1)$$

where the index i is the number of fluorine atoms incorporated with n equal to the maximum number of possible fluorine atoms, $\alpha_i = i/n$, and f_i is the mole fraction of each species represented by i. Thus, peaks resolved from the mass spectrum of a three-Trp protein could have α_i values of 0, 0.33, 0.66, or 1. If all four peaks are of equal height, each mole fraction is 0.25, and the calculated incorporation of 5F-Trp in the total protein is 50%.

Effect of 5-fluorotryptophan on cofactor activity of sTF and mutants

Cofactor activation of VIIa by 5F-Trp labeled wild type and mutant proteins was measured by chromogenic assay based on production of Xa by the cofactor:VIIa complex as previously described (Hasselbacher et al., 1995a). The different cofactor complexes exhibited similar catalytic

efficiencies (± 20%). The apparent equilibrium dissociation constant, K_d, for each protein was derived from fitting enzymatic titrations of VIIa as a function of total cofactor concentration, [cofactor$_{total}$], by least squares, using the appropriate root of the quadratic equation

$$[\text{cofactor:VIIa}]^2 - [\text{cofactor:VIIa}](K_d + [\text{VIIa}_{total}] + [\text{cofactor}_{total}]) + [\text{VIIa}_{total}][\text{cofactor}_{total}] = 0 \quad (2)$$

where the equilibrium concentration of complex, [cofactor:VIIa], is defined by the product of the total VIIa concentration and the ratio of the observed velocity to the maximum velocity when VIIa is saturated with cofactor, $[\text{VIIa}_{total}] \times (V_{obs}/V_{max})$.

The complexes formed by mutants W14F and W158F were indistinguishable from those formed with wild type sTF, exhibiting K_d values of about 2 nM. The apparent affinity of mutant W25Y was about half that of wild type and that of W45F was significantly weaker, with a K_d of about 100 nM. While this is less than the value of 20 nM reported previously (Hasselbacher et al., 1995a), titrations with unlabelled W45F under the current assay conditions also gave K_d values of about 100 nM. The salient observation is that Trp replacement with 5F-Trp affects neither the affinity for VIIa nor the cofactor activity of the wild type and mutant proteins.

^{19}F NMR

Magnetic resonance shifts were assigned for each Trp residue by comparing the wild type protein spectrum with spectra of the single Trp replacement mutants. ^{19}F NMR spectra were obtained at 470 MHz on a Varian Unity 500 at 25°C using a triple resonance probe with the center

proton coil tuned to fluorine. A 12 MHz spectral width, 16 K data points, 60-degree pulse-width, and a relaxation delay of 0.5 seconds were used for data collection. The processing parameters included either 25 or 10 Hz line broadening. The protein samples, in TBS buffer with 10% D_2O (v/v) as the lock solvent, were between 2.5 mg/mL and 20 mg/mL, and 3F-Phenylalanine (3F-Phe) was used as an external standard (-38.0 ppm relative to trifluoroacetic acid). The mole fraction of D_2O varied between 10 and 90% for the SIIS experiments. Stock solutions of 100 mM $GdCl_3$/500 mM EDTA or 100 mM Gd:DPTA (adjusted to pH 7.1) were used for the line broadening experiments, which were performed by sequential addition of the gadolinium complexes.

RESULTS AND DISCUSSION

^{19}F NMR assignments for 5F-Trp residues in sTF

Resonance peaks were assigned for each Trp residue by comparing the spectrum of wild type 5F-Trp sTF with the individual spectra of the single Trp replacement mutants (Figure 2). Four Trp resonances can be identified in the wild type sTF spectrum. There are two well-resolved peaks at -45.39 and -47.21 ppm, and a single peak of two overlapping resonances with maxima at -47.87 and -47.95 ppm. The ratio of the integrals corresponding to the three peaks is 1:1:2, indicating that each 5F-Trp residue contributes equally to the signal. The resonance associated with each 5F-Trp residue was identified by observing which peak in the wild type spectrum was eliminated in the spectrum of each single Trp-replacement mutant. The spectra of three mutants (W14F, W45F, and W158F) were sufficient to assign all four resonances. Since the ratio of the integrals of the peaks in the spectra for mutants W14F and W45F are 1:1:1 (Figure 2), it is evident that the overlapping

resonances in the wild type and W158F spectra must be due to Trp 14 and Trp 45. In addition, it is evident that the resonance most downfield, near -48 ppm, is due to Trp 14. The loss of the central peak at -47.21 ppm in the W158F spectrum indicates that this missing peak is due to Trp 158. Accordingly, the remaining peak in the most up-field position near -45.4 is due to Trp 25. Finally, the ratios of the peak integrals show that replacement of Trp residues by 5F-Trp is random in the mutants, even though the percent analog incorporation is less efficient than in the wild type protein (Table I).

The effect of Trp-to-Phe mutations on protein conformation

It should be noted that with the exception of mutant W158F, the resonances of the 5F-Trp residues in the mutant spectra are shifted with respect to their wild type frequencies (Figure 2). The shifted 5F-Trp resonances are associated with mutation of the Trp residues in Domain I. For example, the spectrum of W14F shows about 0.3 ppm shifts down-field for the 5F-Trp 25 resonance and up-field for the 5F-Trp 45 resonance. By contrast, the resonance associated with 5F-Trp 158, at -47.21 ppm, is the only resonance originating from Domain II, and it is unperturbed by the Trp replacements in Domain I.

The chemical shifts of ^{19}F are influenced strongly by van der Waals contacts and electrostatic interactions (Gerig, 1994; Danielson & Falke, 1996). Hence it is likely that structural rearrangements in the local environment of a 5F-Trp residue will perturb the intrinsic chemical shift. The mutation of Trp-to-Phe will tend to favor structural rearrangements because the phenyl

ring occupies less volume than the indole ring, there is a substantial decrease in dipole moment, and the possibility of hydrogen bond formation is eliminated (Huang et al., 1997). It is evident from the x-ray crystal structures of sTF (Muller et al., 1994; Muller et al., 1996; Harlos et al., 1994) that the local environments of the three Trp residues in Domain I share important elements of structure. For example, the Leu 23 side chain is sandwiched between the aromatic rings of Trp 14 and Trp 25. Also, the Ala 73 main chain atoms abut the aromatic ring of Trp 45, while its methyl side chain abuts the aromatic ring of Trp 14 and the methyl groups of the Leu 23 side chain. Thus, mutation of any one of the three Trp residues in Domain I is likely to perturb the local environments of the other two. However, the fact that the single Trp replacement mutants all form functional complexes with VIIa indicates that the structural perturbations of sTF are sufficiently subtle that the essential features of the wild type cofactor interaction with VIIa are maintained.

Solvent accessibility and the local Environments of the Trp Residues

Based on the difference absorbance and fluorescence spectra of sTF and the single Trp-replacement mutants (Hasselbacher et al., 1995a), the Trp 45 and Trp 158 indole rings are partially exposed to solvent. By comparison, the Trp 14 and Trp 25 indole rings are essentially buried in the protein matrix. Since Trp 45 and Trp 14 are the dominant fluorescence emitters, their relative solvent accessibilities could be assessed by their Stoke's shifts and by quenching using iodide ion. Since Trp 25 and Trp 158 are essentially nonfluorescent, conclusions regarding their local environments were derived only on the basis of their difference absorption spectra. The difference absorption spectra of Trp 25 and Trp 158 are red-shifted compared to the spectrum of either

tryptophan or N-acetyl-tryptophanamide in neutral pH buffer and more similar to the absorption of these model amino acids in dioxane, indicating that the indole rings are largely shielded from solvent. In addition, the vibrational bands of Trp 25 are well resolved, like those of Trp 14, indicating that the side chains of both of these residues are in highly restricted environments. While the spectral shift and resolution of the vibrational bands of each residue's absorption spectrum provide information about the local environment, they are much less sensitive than the changes in the fluorescence properties. Nevertheless, taken together, the difference fluorescence and absorption spectra suggest that the four Trp residues can be separated essentially into two classes in terms of solvent exposure. According to this classification, Trp 14 and Trp 25 are considered "buried" residues, essentially inaccessible to bulk solvent, while Trp 45 and Trp 158 are considered "partially buried" residues, partially buried in the protein matrix and partially exposed to bulk solvent.

The ^{19}F NMR spectrum of wild type sTF reveals chemically distinct local environments for each 5F-Trp residue. Since the individual resonances of the 5F-Trp residues are resolved, it is possible to assess directly the relative solvent exposure of each residue either by observing the degree of solvent-induced isotope-shift, using D_2O, or by measuring the line broadening due to interaction with paramagnetic metals. For a fully solvent-exposed 5F-Trp resonance, the solvent-induced isotope-shift is 100 Hz (Luck and Falke, 1991a). Interaction with paramagnetic ions, by comparison, provides a measure of accessibility to larger solutes. For example, Luck and Falke (1991b) used Gd:EDTA perturbation of a 5F-Trp residue in the sugar binding site of a galactose-

binding protein to investigate the cleft angle of the ligand binding site. The line broadening of accessible fluorine atoms by paramagnetic metals occurs via spin-spin relaxation, which has an inverse sixth power distance dependence, becoming effective within distances of a few Å.

The solvent-induced isotope-shifts of the 5F-Trp resonances of wild type sTF are shown in Figure 3. None of the residues exhibits a solvent shift characteristic of a fully accessible residue. In this regard the SIIS data provide information complementary to that obtained from the difference absorption and fluorescence spectra. As suggested by the difference absorption spectra, the 5F-Trp residue at position 25 is inaccessible to solvent while that at position 158 is partially exposed. The SIIS data, however, show no clear distinction between 5F-Trp 14 and 5F-Trp 45, which may be due to the overlap of their resonances. Both residues exhibit similar solvent exposure albeit less than 5F-Trp 158 and more than 5F-Trp 25. By contrast, the difference fluorescence spectra and quenching with iodide ion indicate clearly that Trp 45 is more exposed to solvent than Trp 14.

The results from the line broadening experiments correspond closely with those from the SIIS experiments in demonstrating the relative solvent exposure of 5F-Trp 158. Accompanying the titration of wild type sTF with Gd:EDTA, the resolved resonance of 5F-Trp 158 broadens significantly as measured by increased full-width at half-height, while the resolved resonance of 5F Trp 25 and the overlapping resonances of 5F-Trp 14 and 5F-Trp 45 appear to be essentially unaffected (Figure 4A). Based upon the fluorescence data, resonance broadening of 5F-Trp 45 also was expected. The fact that Gd:EDTA carries a net charge of –1 and as a result may not approach

residues in close proximity to a negative charge readily was considered. To determine if charge repulsion was playing a role in the lack of line broadening for the other 5F-Trp residues, especially 5F-Trp 45, the experiment was repeated with Gd:DTPA, which has a net charge of 0. However, as with Gd:EDTA, the only resonance observed to broaden significantly was that of 5F-Trp 158 (Figure 4B), indicating that negatively charged residues were not shielding the other Trp residues from Gd:EDTA. The possibility that the effects of Gd:EDTA on 5F-Trp 45 were being obscured by the overlapping 5F-Trp 14 resonance also was considered. To observe perturbation of each of these resonances separately, Gd:EDTA titrations were made on the W14F and W45F mutant proteins. As observed for wild type, only the resonance of 5F-Trp 158 underwent substantial line broadening (Figures 4C and 4D), indicating that the *fluorine* atom of 5F-Trp 45 is not solvent accessible. Therefore, while the difference fluorescence spectrum and fluorescence quenching of Trp 45 show that the indole ring is partially solvent accessible, the lack of ^{19}F NMR line broadening by paramagnetic metals identifies that part of the indole ring which is buried in the protein matrix. As shown in Figure 5, position 5 on the indole ring of Trp 45 is essentially buried in the matrix of the protein, largely shielded from solvent, while the corresponding ring position of Trp 158 is largely exposed to solvent.

The solvent accessibility of Trp 14 and Trp 45, as measured by fluorescence quenching, correlates well with the solvent accessibility area of the indole ring calculated from the x-ray crystal structure (Hasselbacher et al., 1995a). The SIIS and paramagnetic line broadening results, on the other hand, do not show such a direct correlation with the solvent accessible area. The fact that ^{19}F

NMR assesses the local environment of the fluorine atom while absorption and fluorescence assess local environment of the entire indole ring appears to account for the observed differences between the results from difference absorption and fluorescence spectroscopies *versus* ^{19}F NMR spectroscopy.

The utility of combining ^{19}F NMR spectroscopy with fluorescence spectroscopy

We have observed that ^{19}F NMR and fluorescence spectroscopy yield complementary but not identical information on the solvent accessibility of residues in proteins in solution. Fluorescence and NMR probes both are sensitive to perturbation of the local environment. However, ^{19}F NMR spectroscopy makes observations about one atom on a Trp residue while fluorescence spectroscopy makes observations about the average environment of the entire side chain. The sensitivity of ^{19}F NMR spectroscopy to subtle alterations in the local environments of the side chains of the Trp residues is evident from the observed shifts in the resonance peaks associated with Domain I Trp residues in the W14F and W45F mutants. Banner et al. (1996) indicated that the sTF main chain backbone does not experience structural alterations upon binding VIIa. However, this does not presuppose that the side chains do not undergo structural alterations that would facilitate formation of the complex between sTF and VIIa. Thus, we anticipate that ^{19}F NMR spectroscopy will be a useful tool for determining conformational changes in the side chains of sTF resulting from formation of the binary complex with VIIa or ternary complexes with VIIa and ligand or inhibitor.

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Table 1. Percent 5F-Trp incorporation in wild type sTF and single Trp-replacement mutants as estimated by LINCS analysis and by mass spectrometry.

Protein	LINCS	Mass Spectrum
wild-type	100	94/100
W14F	77	74
W25Y	72	68/73
W45F	83	73
W158F	79	74

Percent incorporation of 5F-Trp in mutant and wild type proteins was determined as described in Materials and Methods. Estimated precision of values obtained from LINCS is ± 8 %. Values obtained from mass spectrometry represent results of individual measurements.

Figure Legends:

Figure 1: Rasmol (Sayle & Milner-White, 1995) view of the sTF:VIIa complex based on the 2.0 Å resolution x-ray crystal structure by Banner et al. (1996)). The light gray ribbon indicates the main chain of sTF, and the four Trp side chains of sTF are shown as space-filling representations. The dark gray ribbon indicates the light and heavy main chains of VIIa, and the bound calcium atoms are represented as white spheres. There are seven calcium atoms bound to the Gla domain, one to the EGF domain, and one to the catalytic domain.

Figure 2: ^{19}F NMR spectra (470 MHz, proton-decoupled) of wild type, W158F, W45F and W14F sTF, obtained as described in **Materials and Methods**. The sample buffer was TBS (pH 7.4), 1 mM EDTA, and 1 mM NaN_3, with 10% D_2O as the solvent lock. 3F-Phenylalanine was used as an external standard (-38.0 ppm relative to trifluoroacetic acid).

Figure 3: Solvent-induced isotope-shifts for 3F-phenylalanine (5 mM) and the 5F-Trp resonances of wild type sTF (~ 100 μM). The frequency shifts caused by increasing the mole fraction of D_2O were measured for 5F-Trp 14 (■), 5F-Trp 25 (●), 5F-Trp 45 (▲), and 5F-Trp 158 (◆). The sample buffer was TBS (pH 7.4), 1 mM EDTA, and 1 mM NaN_3.

Figure 4: Perturbation of ^{19}F NMR spectra by paramagnetic metals: Titration end points of wild type sTF (A) with 0 or 7.7 mM Gd:EDTA$^{(-1)}$ and (B) with 0 or 7.6 mM Gd:DPTA$^{(0)}$. Titration

end points of (C) W14F with 0 or 7 mM Gd:EDTA$^{(-1)}$, and (D) W45F with 0 or 7 mM Gd:EDTA$^{(-1)}$.

Figure 5: Rasmol (Sayle & Milner-White, 1995) space-filling representation of the local environments of Trp 45 and Trp 158 in sTF based on the 1.7 Å resolution x-ray crystal structure by Muller et al., (1996). The protein atoms within 10 Å of the C-5 atom of each indole ring are shown in gray. The indole ring atoms are shown in white except for the fluorinated atom C-5, which is black. The arrow indicates the location of the fluorine atom (F) on each indole ring. It should be noted that C-5 of Trp 45 is essentially buried in the protein matrix, while C-5 of Trp 158 is essentially exposed to solvent.

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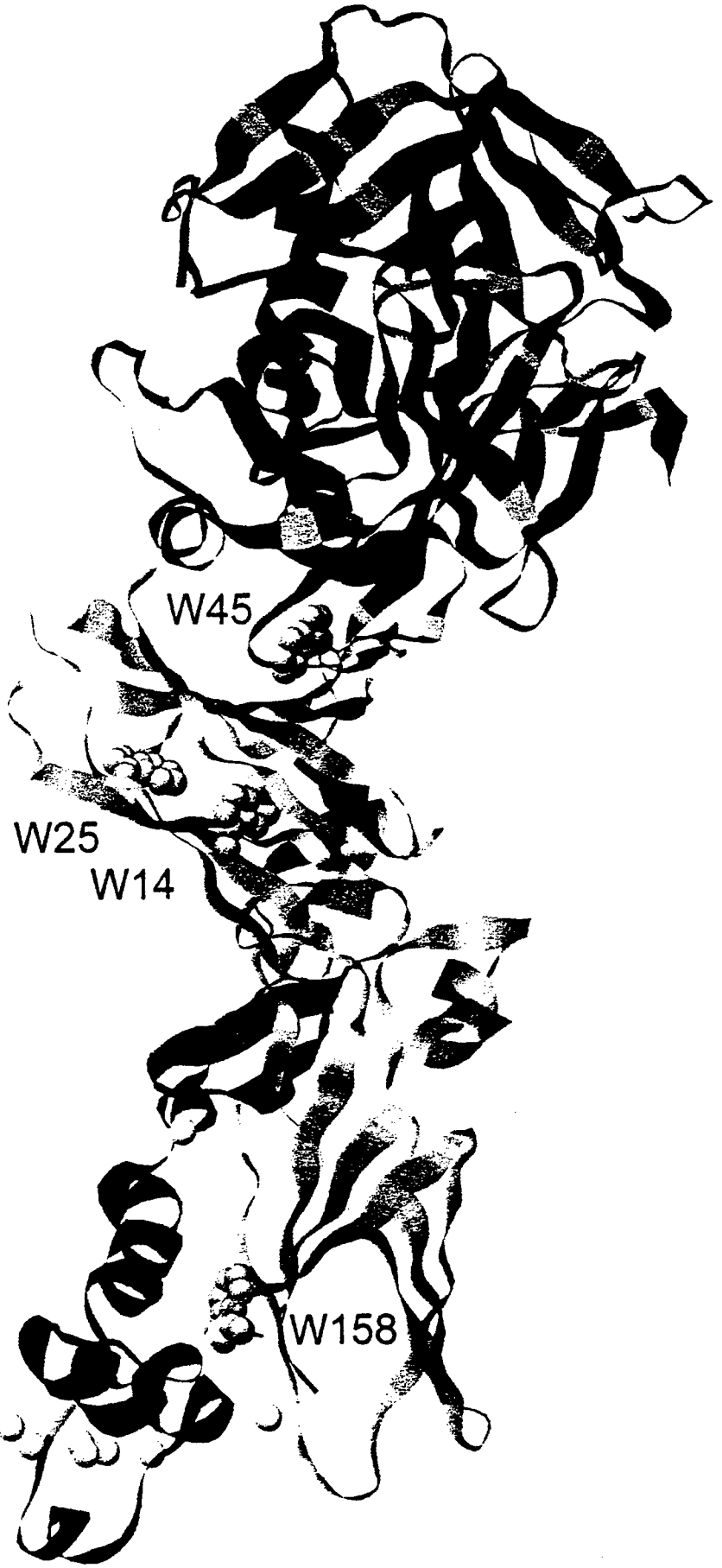
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W25
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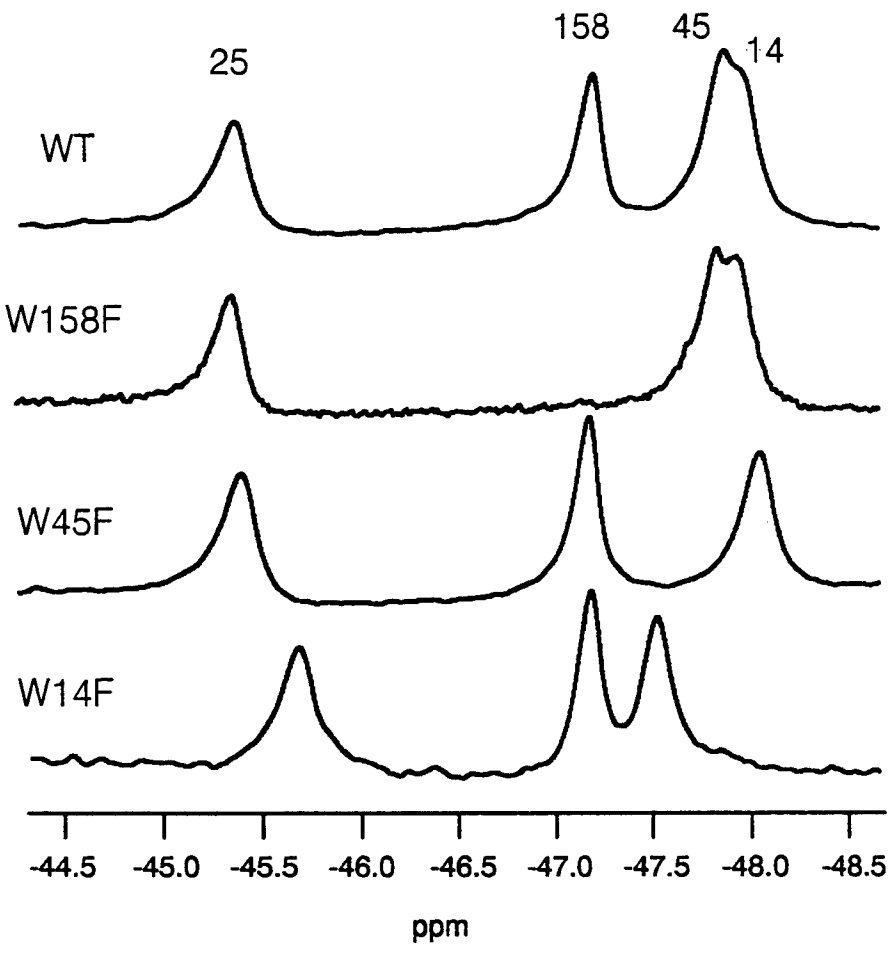
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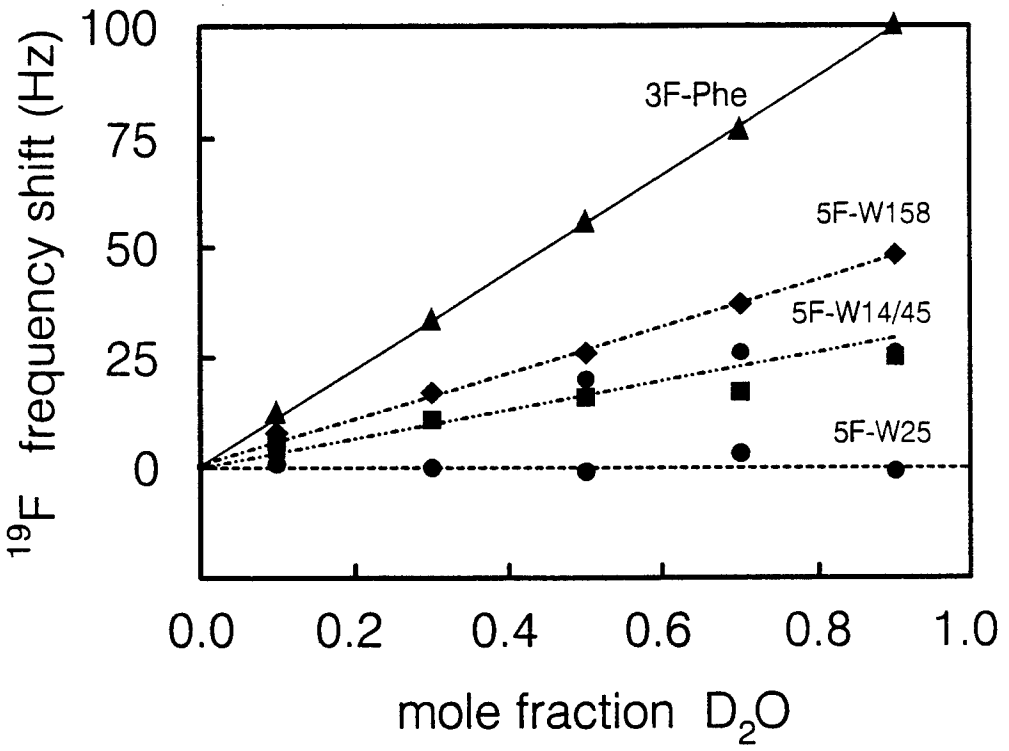
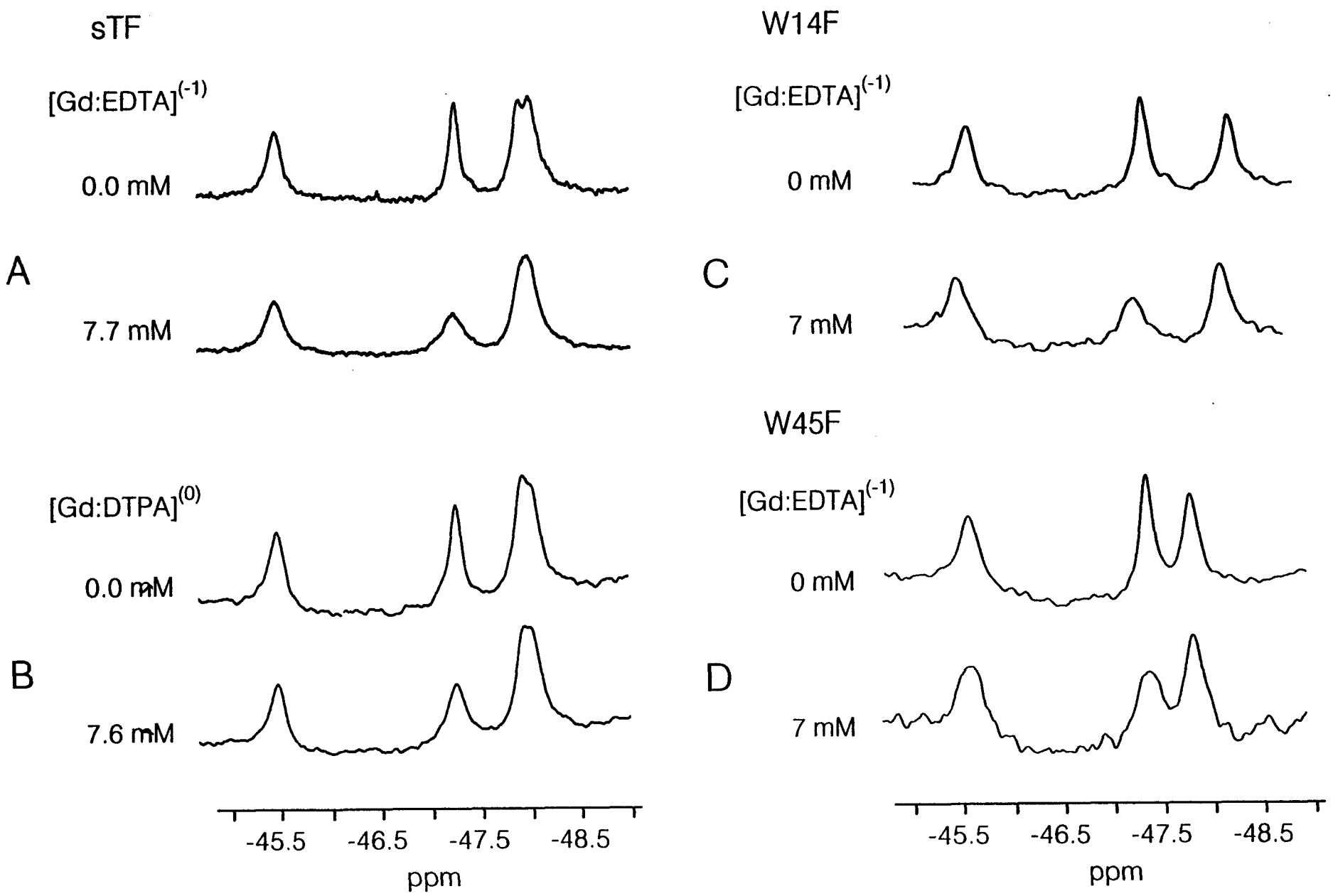
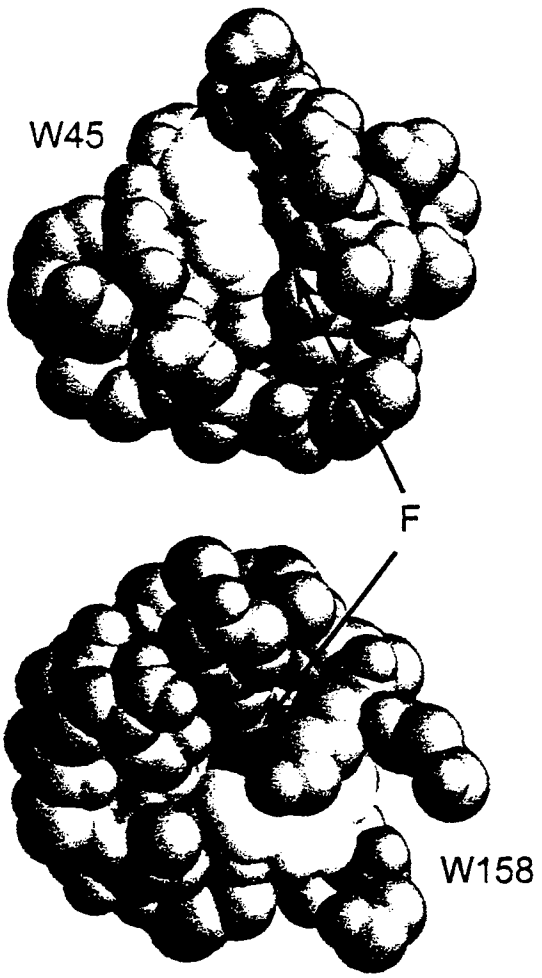
-48.5
ppm


100
75
50
25
0
^{19}F frequency shift (Hz)
3F-Phe
5F-W158
5F-W14/45
5F-W25
0.0
0.2
0.4
0.6
0.8
1.0
mole fraction D_2O


sTF
[Gd:EDTA](-1)
0.0 mM
A
7.7 mM
[Gd:DTPA](0)
0.0 mM
B
7.6 mM
-45.5
-46.5
-47.5
-48.5
ppm
W14F
[Gd:EDTA](-1)
0 mM
C
7 mM
W45F
[Gd:EDTA](-1)
0 mM
D
7 mM
-45.5
-46.5
-47.5
-48.5
ppm


W45
F
W158

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 19 Jan 01

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grant DAMD17-96-1-6140. Request the limited distribution statement for Accession Document Number ADB238997 be changed to "Approved for public release; distribution unlimited." This report should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by email at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management